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14. ABSTRACT
This project tests the hypothesis that inherited and/or acquired differences in telomere length unprostate cancer observed in African Americans compared to Caucasians and in affected members of hefamilies. To test this hypothesis, telomere content in genomic DNA isolated from blood samples of by quantitative PCR and the data assessed for associations with either racial background or prostathe period of this report we have isolated genomic DNA from 128 members from affected and unaffectBuffy coat has been isolated from anonymous cord blood donor samples collected from newborns of diDNA has been isolated form these samples. We are currently still awaiting receipt of the mid-life Health Professionals Follow-up Study based at Harvard University. Analysis of the telomere lengthsgenomic DNA samples reveals that telomere lengths are shorter in affected compared with non-affecta statistically significant difference (p=0.026). This result supports the hypothesis that telomerdevelopment of prostate cancer within families displaying elevated risk.

15. SUBJECT TERMS
Telomere, Prostate Cancer, Health disparity, Familial prostate cancer, Carcinogenesis

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Introduction

The overall goal of our research is to determine the role played by telomere biology in human prostate cancer. In this proposal we are examining the hypothesis that telomere length abnormalities play a causal role in prostate carcinogenesis. Accordingly, we are testing this in two populations enriched for prostate cancer - African American men, and members of hereditary prostate cancer (HPC) families. If the hypothesis is correct, we expect to find (i) significantly shorter telomeres in African American males versus age-matched Caucasian males and/or (ii) an association between shorter telomeres in affected members of prostate cancer families versus age-matched unaffected relatives. Such support for the hypothesis would highlight the importance of telomere biology in prostate cancer, thus future studies in this area could lead to new targets for the prevention and treatment of the disease. Furthermore, a defined link between telomere length, as measured in peripheral blood samples, and prostate cancer risk could lead to a simple blood test to aid in the assessment of a man's risk of developing prostate cancer in high risk settings and potentially in the general population as well.

Body

Summary of timeline: This PCRP New Investigator Award had an original duration of two years and a December 01, 2005 start date, with the included provision that approval for the use of human subjects was still pending at that time. Final written authorization for the use of human subjects material was received one year later, thus there was a one year delay in initiating the research as it utilizes human-derived blood and DNA samples. Therefore, during Year 1 we procured supplies and established general quantitative real-time PCR (Q-PCR) methodology in the lab. By Year 2 we had also acquired local IRB human subjects approval, as well as approval from the other institutions from which samples were to be obtained. This delay represented one of the problems we encountered in conducting the research project.

During Year 2 we did extensive optimization and testing of the Q-PCR method for quantifying telomere DNA content in our lab and validated it using DNA derived from cells with known telomere lengths, which we independently measured by a Southern blot assay. Further assay validation was conducted using a DNA dilution series of genomic DNA isolated form cultured normal human cells as well as blood-derived test DNA (data presented in Year 2 progress report). We encountered significant difficulty in implementing the telomere Q-PCR content assay and this represented a second major hurdle we encountered in conducting the research project. As a backup plan, we began developing a novel quantitative telomere PCR assay based on padlock probe technology. This method was presented at the 2007 DOD IMPACT Meeting in Atlanta, Georgia. In addition to technical issues with the Q-PCR assay, we also had difficulty obtaining the biological samples from two of our three sources in Year 2. Although blood samples were sent from the Johns Hopkins School of Public Health they were lost in transit. A second set of samples was received but turned out to be an incorrect sample set and had to be returned. The request for buffy coat samples from the Health Professionals Follow-up Study (HPFS), based at Harvard University, remained in queue through Year2. Samples were procured and genomic DNA purified and quantified from 128 affected and non-affected individuals from the Johns Hopkins Familial Prostate Cancer Registry based in the Department of Urology. Thus sample procurement represented another problem we encountered in conducting our research. During Year 2 it became clear that additional time would be required to complete our studies, thus we applied for and were granted a one year no-cost extension.

During Year 3 we finished optimizing, testing and validating the telomere Q-PCR assay. Once we were confident that the assay was performing as required we assayed the genomic DNA samples from our HPC cohort, the results of which constitute the bulk of this report. We have now received all of the correct cord blood samples and are finishing the DNA isolation on these. The HPFS samples are currently being pulled at Harvard; we have been billed for this service and we anticipate their arrival in the near future. Due to this continued delay in sample acquisition we have applied for an additional one year no-cost extension. We are confident that all remaining work can be completed within this 12 month period and we will then file an amended final report to USAMRMC.

Results: Quantitative PCR was used to assess telomere repeat content in genomic DNA samples isolated from 128 members of HPC families. Samples were pulled from 17 different HPC families and included 71 affected men (biopsy-proven prostate cancer) and 57 non-affected family members (22 male, 35 female). Telomere content was measured in triplicate by Syber green Q-PCR performed on 5 ng of genomic DNA (Cawthon, 2002). To correct for differences in DNA concentrations, separate Q-PCR reactions were run, also in triplicate, on each sample for the single copy gene beta-globin. In addition to negative (no template) controls, each plate also included two 5-point dilution series standard curves selected from the same set of genomic DNAs being assayed. These standard curves served as quality control indicators and allowed us to determine the PCR efficiency for each separate experimental run. Furthermore, each run included three genomic DNA samples isolated from LNCaP prostate cancer cell lines with known differing telomere lengths (as determined by Terminal Restriction Fragment analysis; the accepted "gold standard") spanning the expected range of approximately 3 – 15 kilobases (Kbp) of telomere repeats. As well as serving as an additional QC check this sample set allowed us to directly convert our telomere Q-PCR measurements to actual average telomeric DNA lengths, expressed in Kbp. Since different PCR efficiencies were obtained for the telomere and beta-globin PCRs, the Pfaffl method was applied to determine the normalized T/S ratio. For each sample, the following equation was used:

$$\text{T/S Ratio} = (E_{\text{telomere}}{}^{\Delta Ct,\ \text{telomere (calibrator – test)}}) \,/\, (E_{\beta\text{-globin}}{}^{\Delta Ct,\ \beta\text{-globin (calibrator – test)}})$$

In this equation, E is the amplification efficiencies for the telomere and β-globin reactions. Next, the LNCaP standard curve was used to generate a best fit line equation and the normalized T/S ratio was used to determine the telomere length (in Kb) for each experimental sample. All standard curves had an R^2 value >0.99 (range: 0.991-0.998) for both telomere & beta-globin Q-PCR assays. The coefficient of variation for replicates across multiple assays on the LNCaP series ranged from 1.86% to 7.58%, with the higher CVs obtained from those samples with the lowest telomere DNA content, thus also having the smallest values for mean telomere length.

Average telomere lengths for the 128 HPC family members are presented in Table 1. These data are also plotted individually by family as well as in aggregate in Appendices 1-18.

Table 1 Telomere lengths measured by Q-PCR in samples from HPC family members

HPC Family	Mean Telomere Length*	Gender	Race	Disease Status	HPC Family	Mean Telomere Length*	Gender	Race	Disease Status
29	3.51	F	C		84	4.79	M	C	PCa
	5.39	M	C	PCa		5.27	F	C	
	5.41	F	C			6.80	M	C	PCa
	5.53	F	C			7.87	F	C	
	6.57	M	C	PCa		8.39	M	C	PCa
	7.17	M	C			8.41	M	C	PCa
	7.45	M	C	PCa					
	7.55	M	C	PCa	97	4.21	F	AA	
	8.28	M	C			6.07	M	AA	PCa
	8.34	M	C			6.17	M	AA	PCa
	9.25	M	C			6.90	M	AA	PCa
	9.45	F	C			7.52	F	AA	
	9.90	M	C	PCa		8.54	F	AA	
	9.98	M	C	PCa		8.55	M	AA	PCa
	12.51	M	C			8.65	M	AA	PCa
	12.68	M	C	PCa		9.93	M	AA	PCa
	31.72	M	C	PCa					
					113	5.23	F	C	
43	5.68	F	C			5.34	M	C	PCa
	6.11	M	C	PCa		6.18	M	C	PCa
	6.18	F	C			6.19	M	C	PCa
	7.13	M	C	PCa		6.35	M	C	PCa
	8.51	M	C	PCa		7.20	F	C	
	8.82	M	C			7.38	M	C	
	12.53	M	C			7.42	F	C	
	12.73	F	C			7.53	M	C	PCa
						9.26	M	C	
77	3.66	M	C	PCa		9.52	M	C	
	4.33	F	C			9.65	M	C	PCa
	5.45	M	C	PCa		10.10	M	C	PCa
	5.78	M	C	PCa		10.85	M	C	
	6.70	M	C	PCa		11.55	F	C	
	6.85	M	C						
	7.67	M	C	PCa	119	5.83	F	C	
						5.85	M	C	PCa
						6.66	M	C	PCa
						8.39	M	C	PCa
						9.34	M	C	PCa
						11.80	F	C	

Table 1 (continued)

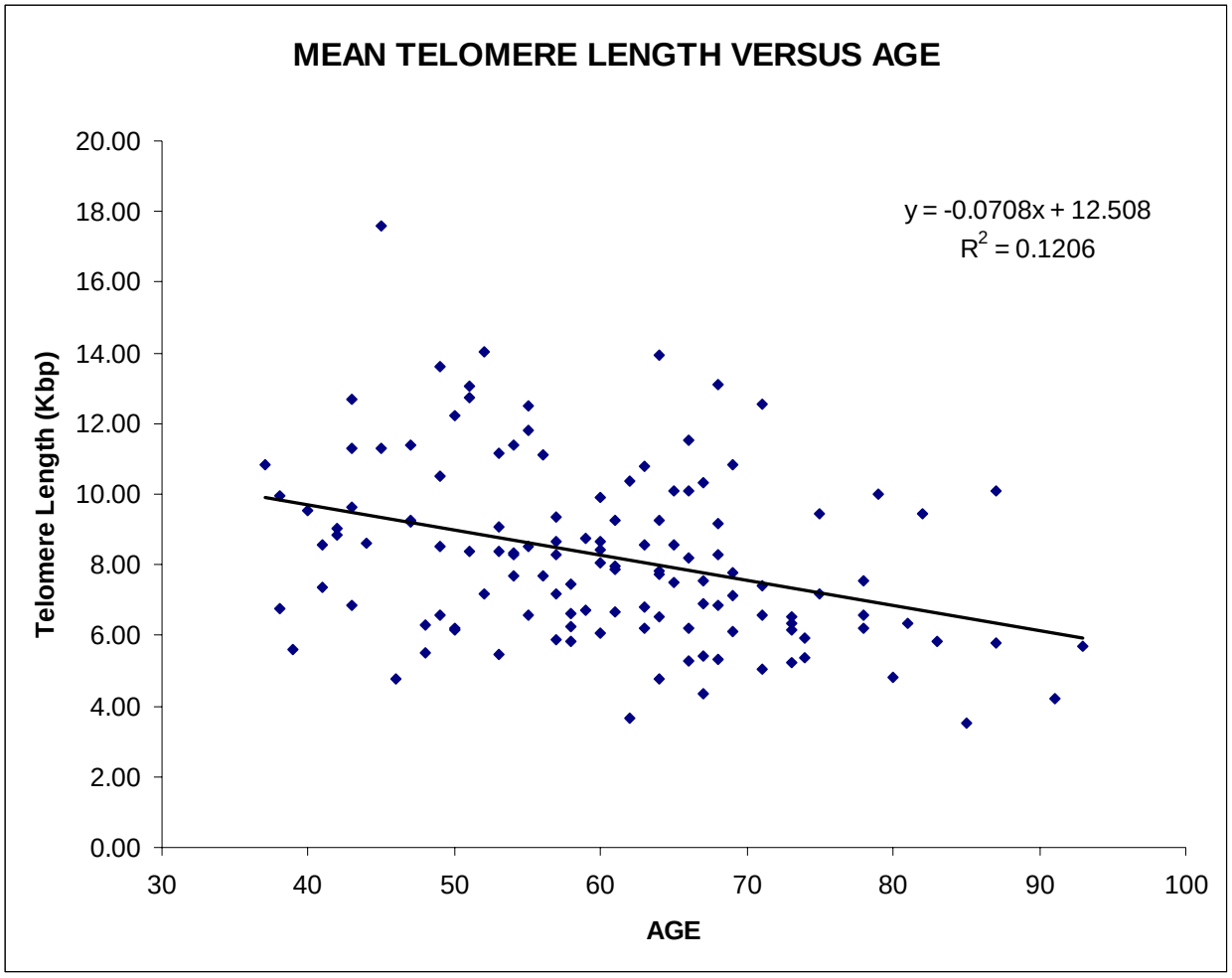
HPC Family	Mean Telomere Length*	Gender	Race	Disease Status	HPC Family	Mean Telomere Length*	Gender	Race	Disease Status
134	6.23	M	C	PCa	231	7.74	M	C	
	6.54	M	C	PCa		7.85	M	C	PCa
	6.60	M	C	PCa		7.98	M	C	PCa
	7.76	F	C			8.05	M	C	PCa
	8.63	M	C			8.66	F	C	
						9.16	F	C	
137	5.59	M	AA	PCa					
	6.59	M	AA	PCa	239	6.36	M	C	PCa
	7.67	M	AA	PCa		9.43	M	C	PCa
	8.18	M	AA	PCa		11.18	M	C	PCa
	9.05	M	AA			11.37	M	C	PCa
	11.29	M	AA			12.23	F	C	
						14.04	M	C	PCa
210	4.79	M	C	PCa		17.58	F	C	
	4.82	M	C						
	5.06	M	C	PCa	241	8.74	M	C	PCa
	6.22	M	C			10.08	F	C	
	6.31	F	C			10.11	M	C	
	6.53	F	C			10.39	M	C	PCa
	6.76	F	C			10.85	F	C	
	6.85	M	C	PCa		11.09	F	C	
						11.38	F	C	
214	5.87	M	C	PCa		13.07	F	C	
	5.91	F	C			13.12	M	C	PCa
	7.20	M	C	PCa		13.63	F	C	
	8.29	M	C	PCa		13.93	F	C	
227	8.57	M	C	PCa	244	6.19	M	C	PCa
	9.25	M	C	PCa		6.56	M	C	PCa
	10.33	M	C	PCa		6.59	M	C	PCa
	10.79	M	C	PCa		8.31	M	C	PCa
						9.06	M	C	
230	8.55	M	C						
	9.22	M	C	PCa					
	10.51	M	C	PCa					
	11.29	M	C	PCa					

*Telomere lengths listed are averages of at least 3 separate measurements.

Abbreviations: M = Male, F= Female, C= Caucasian, AA= African American, PCa= Prostate Cancer.

In figure 1, telomere length data is plotted as a function of donor age for the entire cohort, excluding the last individual in family #29 who's telomere length was >6 standard deviations away form the overall mean for the cohort and was thus considered an outlier. The observed range of telomere lengths observed, the broad inter-individual variation in telomere lengths and the slight decline in telomere length with increasing age are wholly consistent with previously published series in other studies on telomere lengths in human subjects.

Figure 1



Analysis of the telomere length data for the entire cohort reveals that average telomere lengths are shorter in affected compared with non-affected male members of HPC families (Table 2). This difference was statistically significant (p=0.026).

Table 2 Telomere lengths in non-affected and affected individuals of 17 HPC families (n=128)

	Non-affected	Affected
Mean	8.87	7.86
SD	1.92	2.10
Median	8.82	7.55

Significance: Telomeres are critical structural DNA elements of chromosomes that serve to maintain chromosomal integrity. Telomere dysfunction, particularly due to loss of telomere repeats, results in genomic instability which can, in turn, foster malignant transformation. We and others have previously published data indicating that telomere shortening is a common characteristic of cancer cells and, notably, most pre-malignant lesions as well, thus supporting a causal role for telomere shortening in human carcinogenesis. In the project described herein, we have utilized a PCR-based approach to examine the telomere length status in genomic DNA isolated from normal blood lymphocytes; thought to serve as a proxy for an individual's constitutional telomere length. The results presented here on members of hereditary prostate cancer families indicate that telomeres are shorter in affected individuals compared to their non-affected relatives. This implies that telomere shortening may, at least in part, help explain the increased risk for prostate cancer in these families. This is notable in that although these families clearly possess some heritable defect that puts them at elevated risk, linkage studies have failed to uncover any strong association to specific genomic loci. We hypothesize that this may be explained by the fact that the heritable entity is not a gene, but rather telomere length (a known heritable trait); a chromosomal element that standard genomic analyses are totally blind to. It is conceivable that not all families in our study have an increased prostate cancer risk due to inheritance of shorter telomeres. In this regard it is worth noting that certain families (e.g. families 43, 134, 137, 231, 239 and 244) display a higher degree of skewing for affected individuals towards shorter telomeres. The affected individuals in these families in particular may have inherited relatively shorter telomere lengths and it will be interesting to assay additional individual's samples from these families to explore this possibility further.

The magnitude of the telomere length difference between affecteds and non-affecteds we observed was approximately 1 Kbp (1,000 base pairs). It is quite plausible that this difference has biological relevance, given that telomere lengths are known to be quite heterogeneous, both between different cells as well as among the 92 chromosomal ends present within each cell. Therefore it is possible that losses of telomeric DNA on the order of 1 Kbp could serve to cause telomeres within the shorter range of the distribution to become destabilized, thus instigating genomic instability with its inherent risk for tumorigenesis.

Key Research Accomplishments

- Q-PCR telomere content assay validated using control genomic DNA dilution series and genomic DNA from cell lines having known telomere lengths, thus allowing determination of average telomere lengths in kilobase pairs.
- Assessment of telomere lengths from blood-derived genomic DNA for 128 members of 17 hereditary prostate cancer families.
- Identification of a statistically significant difference in telomere lengths between affected and unaffected HPC family members; telomeres being approximately 1Kbp shorter in affected individuals.

Reportable Outcomes

A. As a result of the support and experience received through the DOD New Investigator Award, support for additional projects related to telomere biology in prostate cancer was obtained:

Department of Defense W81XWH-05-1-0030 (Elizabeth Platz, PI)
11/01/2004-10/31/2008
Telomere Length as Predictor of Aggressive Prostate Cancer
The goals of this project are to evaluate whether telomere shortening predicts aggressive prostate cancer in cohort of men and to determine whether dietary and lifestyle factors that influence cellular proliferation or oxidative stress predict telomere length in normal appearing prostate and in peripheral blood lymphocytes.
Role: Co-Investigator

NIH/NCI P01CA108964-01A1 (Project 4; Elizabeth Platz, PI)

05/01/2005-04/30/2010
Genotypic and Phenotypic Studies of Inflammation in the PCPT
The goal of this project, which is a component of the program project entitled “Biology of the Prostate Cancer Prevention Trial (PCPT)” is to examine the contribution of the extent of intraprostatic inflammation and atrophy as assessed in biopsies, polymorphisms in genes involved in inflammation and response to infection, and presence of antibodies against infectious agents to prostate cancer.
Role: Co-Investigator

Patrick C. Walsh Prostate Cancer Research Fund
04/01/2007-03/31/2008
Specific Detection of Prostate Cancer in Urine by Multiplex Immunofluorescence and Telomere FISH – Guiding Clinical Decisions Following Negative Prostate Biopsy
The goal of this project is to develop a novel cell-based assay involving simultaneous staining of telomeres and a set of protein molecular markers to allow specific identification of prostate cancer cells in urine cytology specimens.

Role: PI

Patrick C. Walsh Prostate Cancer Research Fund
04/01/2007-03/31/2008
The Senescent Phenotype in Human Prostate Cancer: Pilot Characterization Study and Association with Aging and Cellular Stress
The goal of this project is to characterize the senescent phenotype in the human prostate, its relationship to age, modulation of the phenotype by dietary factors and oxidative damage, and how it relates to risk of prostate cancer.
Role: Co-investigator

B. Experience gained while supported by the DOD New Investigator Award contributed to the following presentations related to telomere biology:

Peer Reviewed manuscripts

1. Palapattu GS, Meeker AK, Harris T, Collector MI, Sharkis SJ, DeMarzo AM, Warlick C, Drake CG, Nelson WG. Epithelial architectural destruction is necessary for bone marrow derived cell contribution to regenerating prostate epithelium. *Journal of Urology.* 176:813-818. 2006. Cover article.

2. Hansel, D.E., Meeker, A.K., Hicks, J., De Marzo, A.M., Lillemoe, K.D., Schulick, R., Hruban, R.H., Maitra, A., Argani, P. Telomere length variation in biliary tract metaplasia, dysplasia, and carcinoma. Modern Pathology. 19:772-779, 2006.

3. Kawai T, Hiroi S, Nakanishi K, Meeker, AK. Telomere length and telomerase expression in atypical adenomatous hyperplasia and small bronchioloalveolar carcinoma of the lung. *American Journal of Clinical Pathology.* 127:254-262. 2007.

4. Stewénius Y, Jin Y, Øra I, de Kraker J, Bras J, Frigyesi A, Alumets J, Sandstedt B, Meeker AK, Gisselsson D. Defective chromosome segregation and telomere dysfunction in aggressive Wilms' tumors. *Clinical Cancer Research*. 13:6593-6602. 2007.

5. Bechan GI, Meeker AK, Marzo AM, Racke F, Jaffe R, Sugar E, Arceci RJ. Telomere length shortening in Langerhans cell histiocytosis. *British Journal of Haematology*. Published article online: 20-Dec-2007.

6. Cummings, S.D., Ryu, B., Samuels, M.A., Yu, X., Meeker, A.K., Healey, M.A., Alani, R.M. Id1 delays senescence of primary human melanocytes. *Molecular Carcinogenesis*. 2008. Epub ahead of print.

Published abstracts from presentations

1. Meeker AK, Vander Griend D, Konishi Y, Isaacs JT. Combined fluorescence in situ hybridization (FISH) for telomeres and centromeres provides rapid and simple discrimination of species of origin for cells in tissue recombination experiments. Modern Pathology21: 366A-367A 1669 Suppl. 1 Jan. 2008

2. Meeker AK, Hicks JL, Smearman E, De Marzo AM. A chromogenic in situ hybridization (CISH) technique for visualizing telomeric DNA in fixed tissue sections. Lab. Invest.86: 331A-331A 1544 Suppl. 1 Jan. 2006

3. Meeker AK, Epstein JI, Konishi Y, Netto GJ. Direct assessment of telomeres in testicular germ cell tumors reveals evidence of telomere length heterogeneity and non-telomerase mediated telomere maintenance in tumor subsets. Modern Pathology 20: 163A-163A 739 Suppl. 2 Mar. 2007.

4. Meeker AK, Bova GS, Hicks JL, De Marzo AM. Direct in situ analysis of telomere lengths in primary tumors and corresponding local and distant metastases obtained via rapid autopsy Modern Pathology 18: 155A-155A 717 Suppl. 1 Jan. 2005

5. Iwata T, Meeker AK, Smearman E, De Marzo AM. The telomere shortening in prostatic atrophy lesions Lab. Invest.88: 162A-162A 738 Suppl. 1 Jan. 2008.

Conclusion

Support for the PI by the DOD New Investigator Award was critical in enabling a successful early career transition from postdoctoral fellow to junior faculty with a primary focus on prostate cancer research. In addition, experience gained through conducting this study has helped in initiating other research projects aimed at elucidating the role of telomeres in prostate cancer.

The results obtained to date support a role for telomere shortening in the development of prostate cancer in hereditary prostate cancer families and suggest that inheritance of chromosomes with reduced telomere lengths may place men at increased risk of developing the disease. Should further study validate this hypothesis it could provide a relatively simple, non-invasive assay to help with risk assessment in members of HPC families.

References

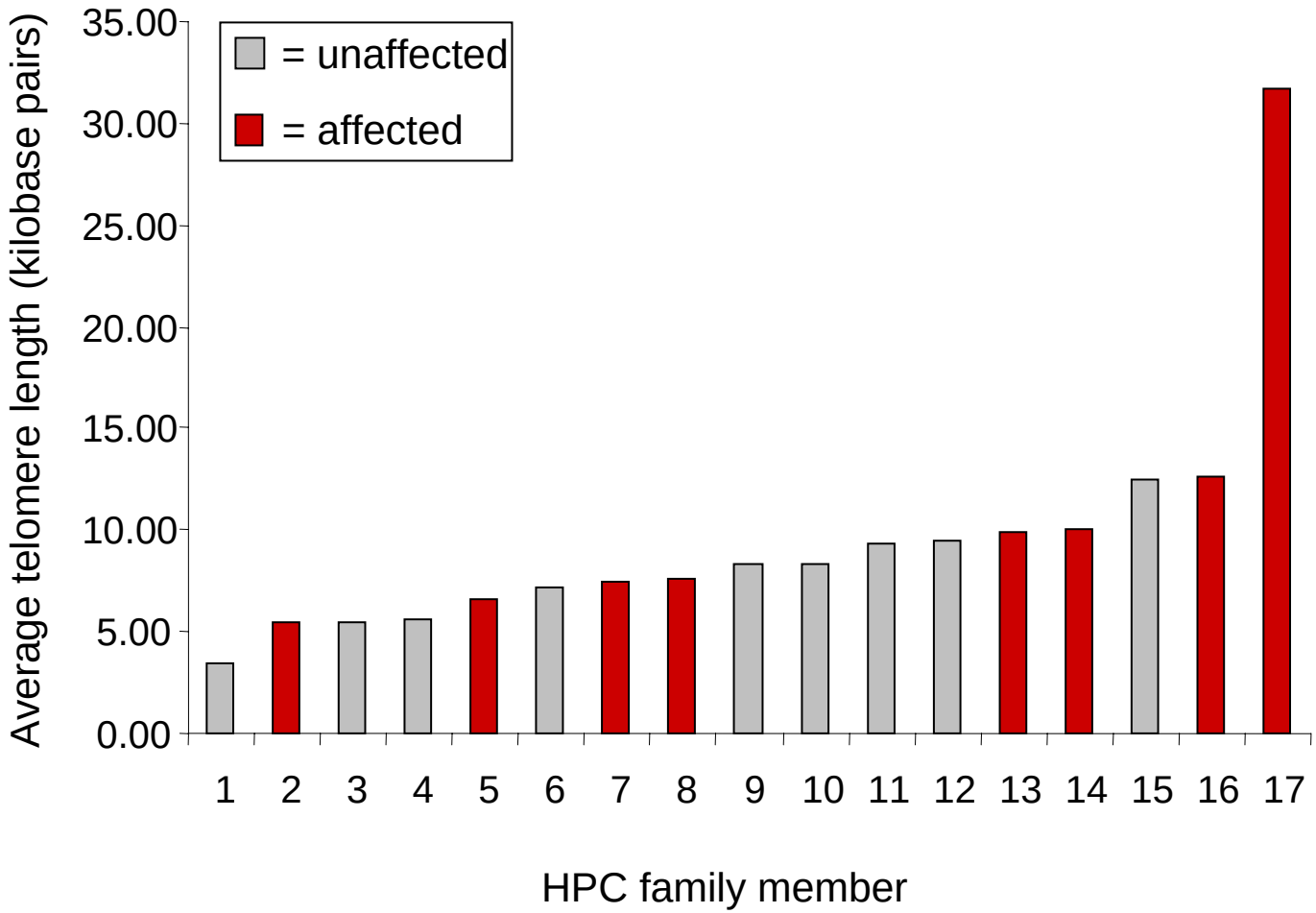
Cawthon, R.M. Telomere measurement by quantitative PCR. Nucleic Acids Res, May 15;30(10):e47, 2002.

Appendices

A1-17: individual plots of telomere length data for each of the 17 HPC families.

A18: plot of telomere lengths for the entire cohort

A1: Family #29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30.00
25.00
20.00
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5.00
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HPC family member

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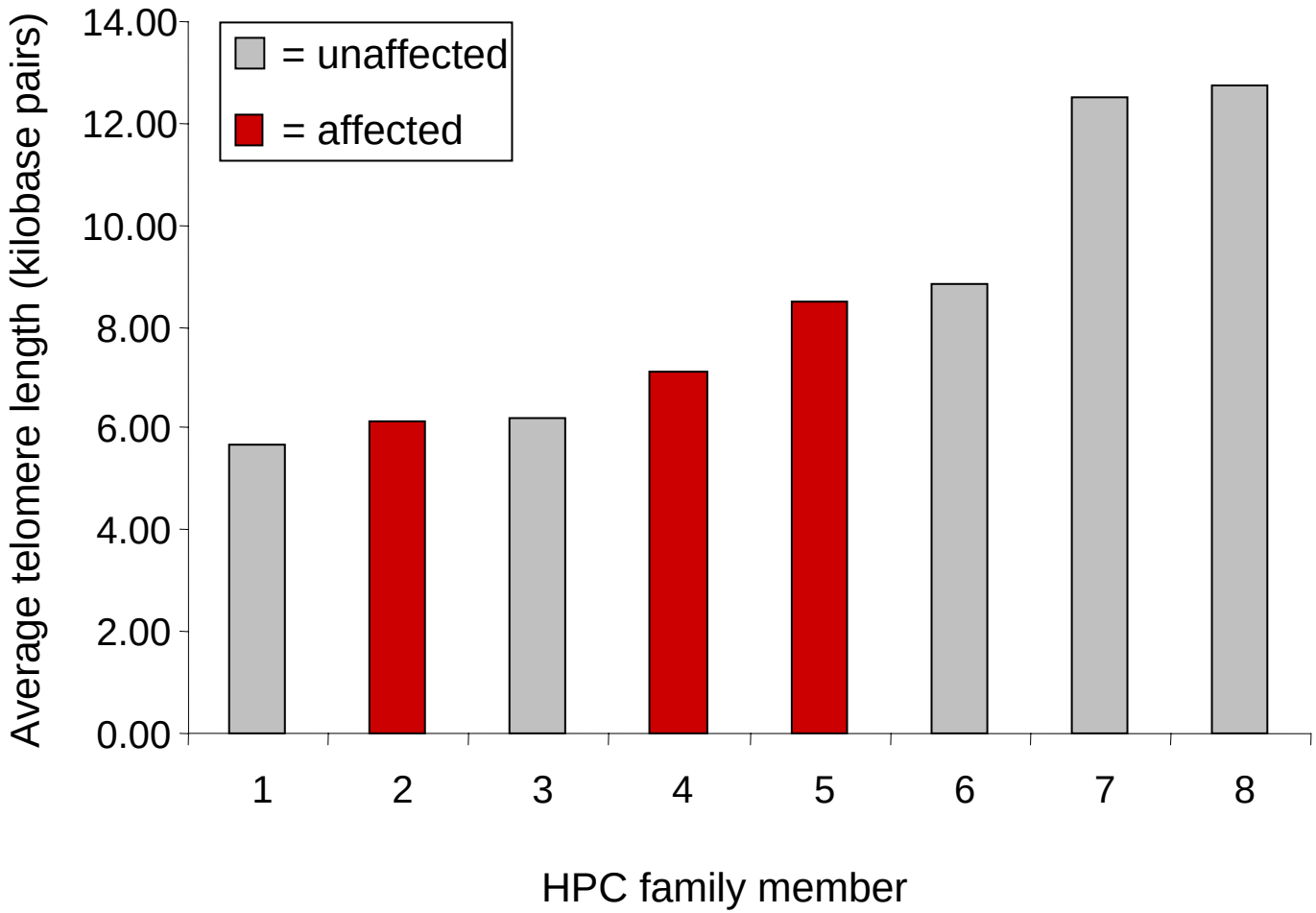
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8.00
6.00
4.00
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3
4
5
6
7
8
HPC family member

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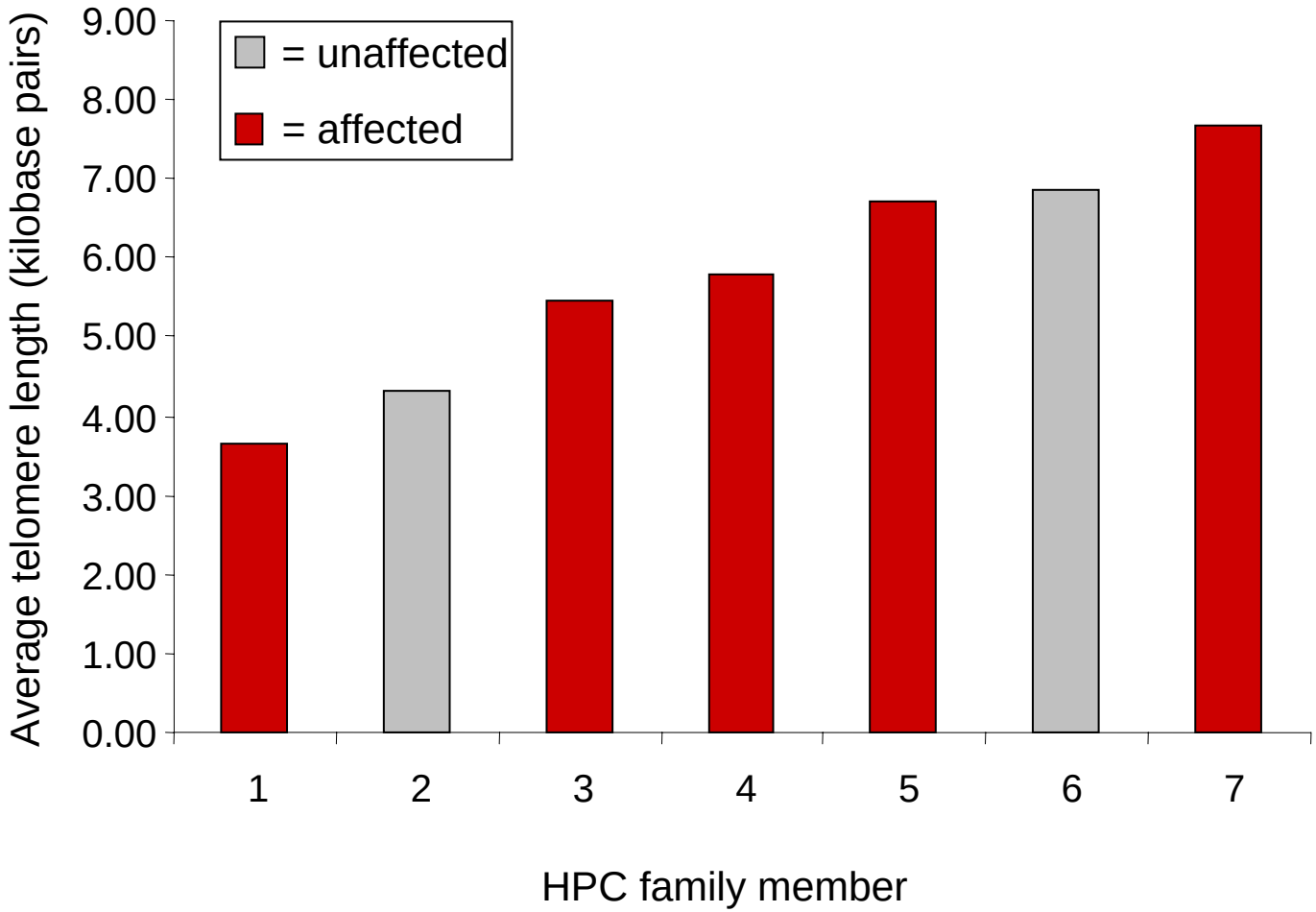
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7.00
6.00
5.00
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1
2
3
4
5
6
7
HPC family member

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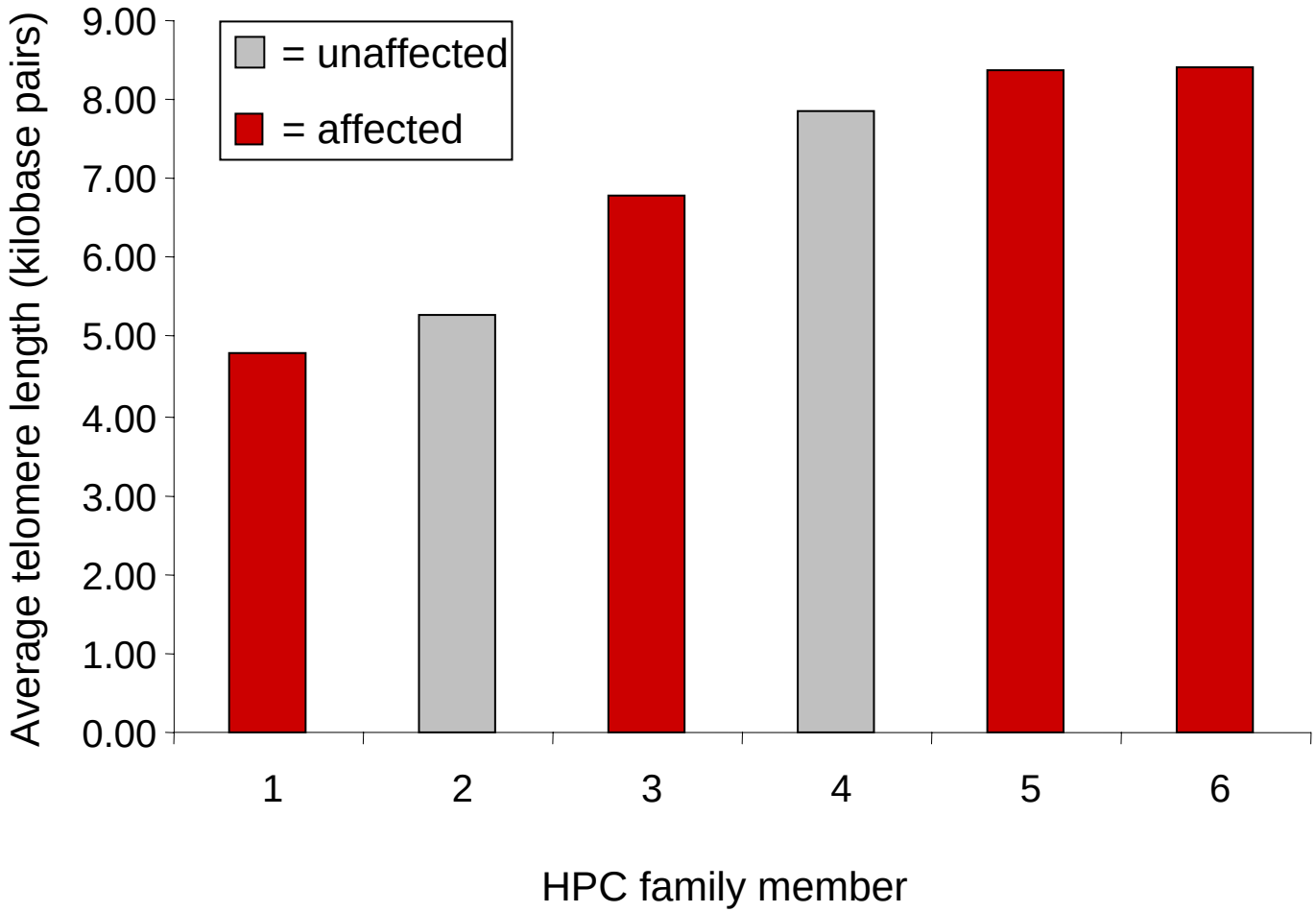
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7.00
6.00
5.00
4.00
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2.00
1.00
0.00
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= affected
1
2
3
4
5
6
HPC family member

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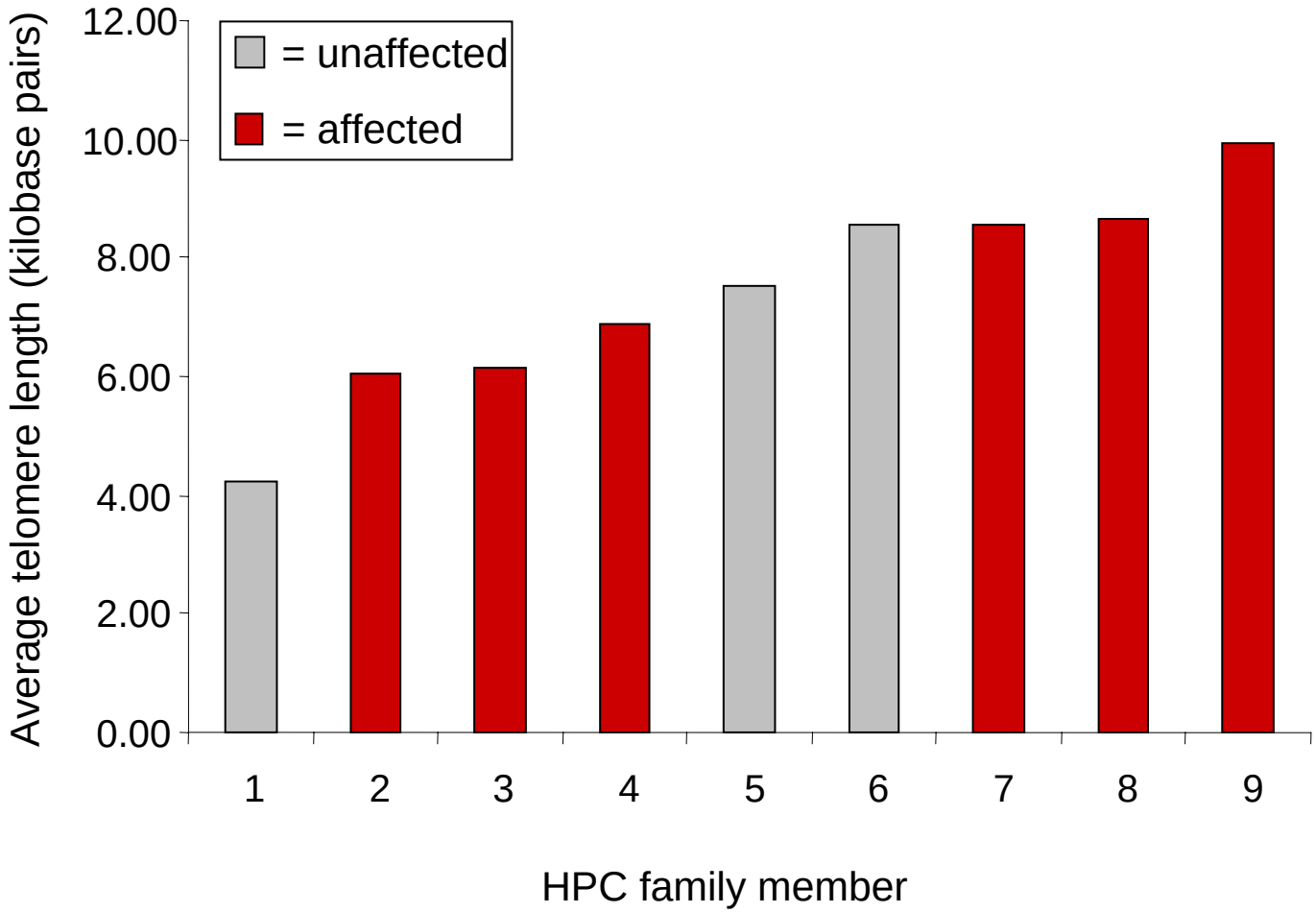
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10.00
8.00
6.00
4.00
2.00
0.00
= unaffected
= affected
Average telomere length (kilobase pairs)
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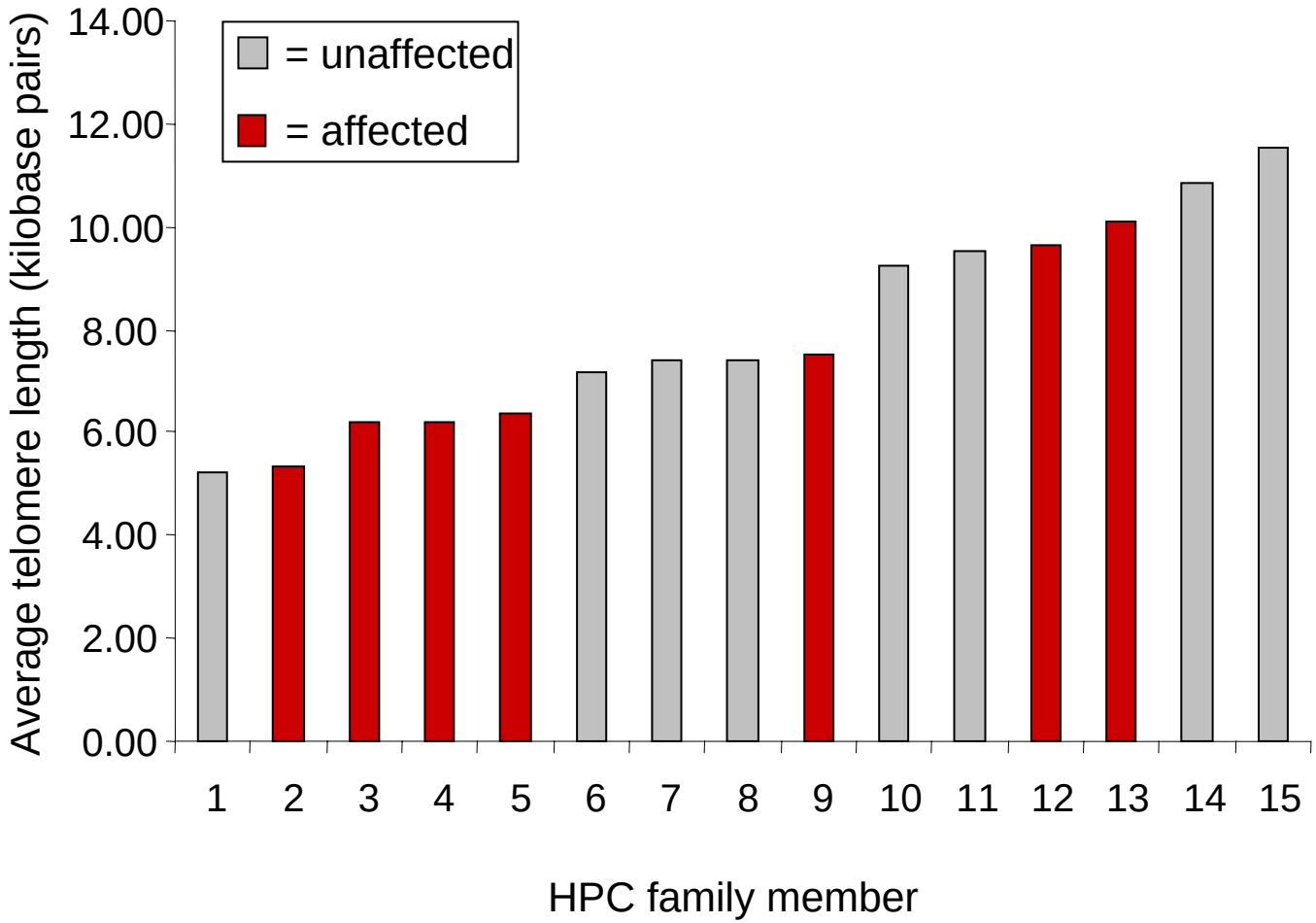
2
3
4
5
6
7
8
9
HPC family member

A6: Family #113



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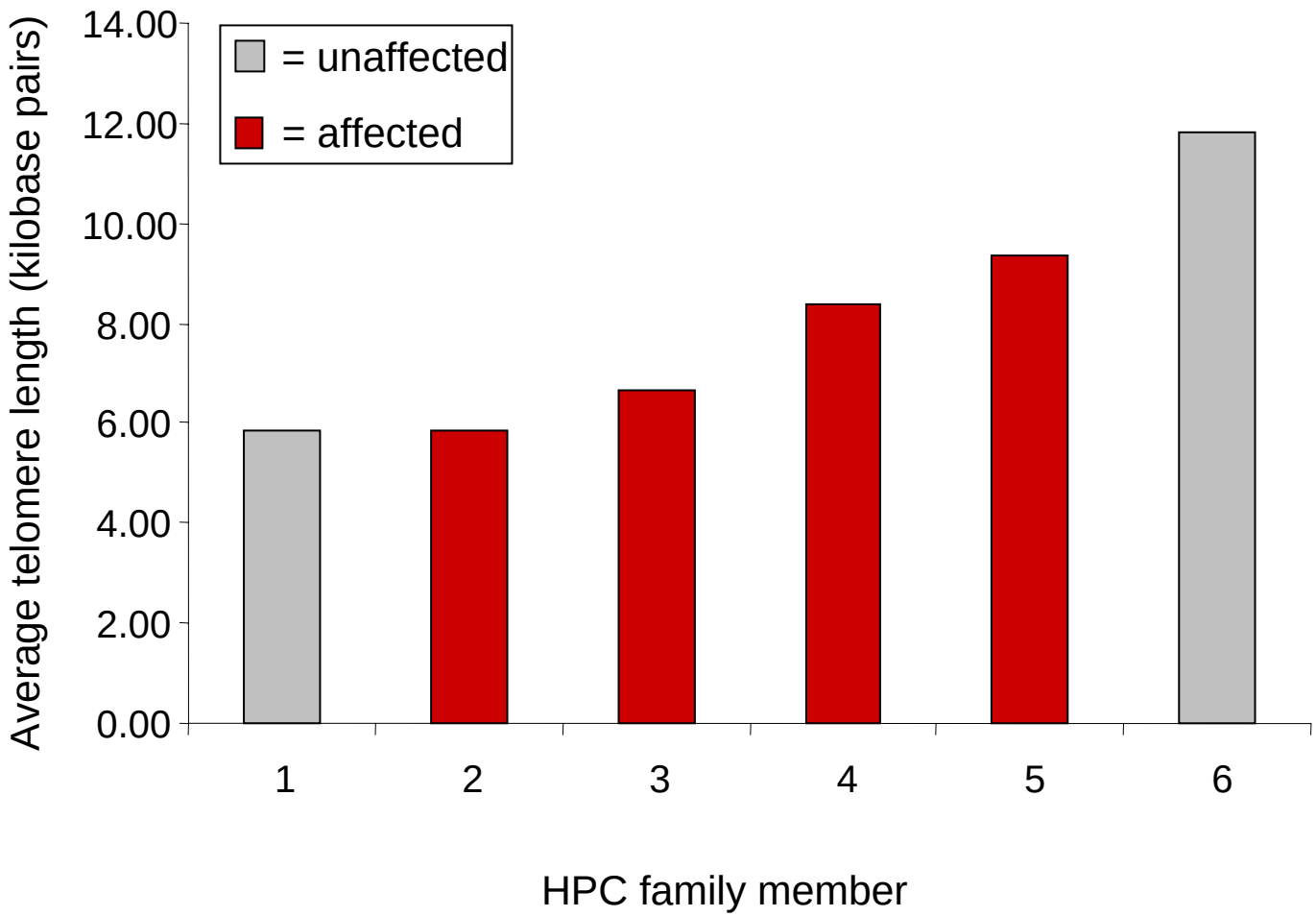

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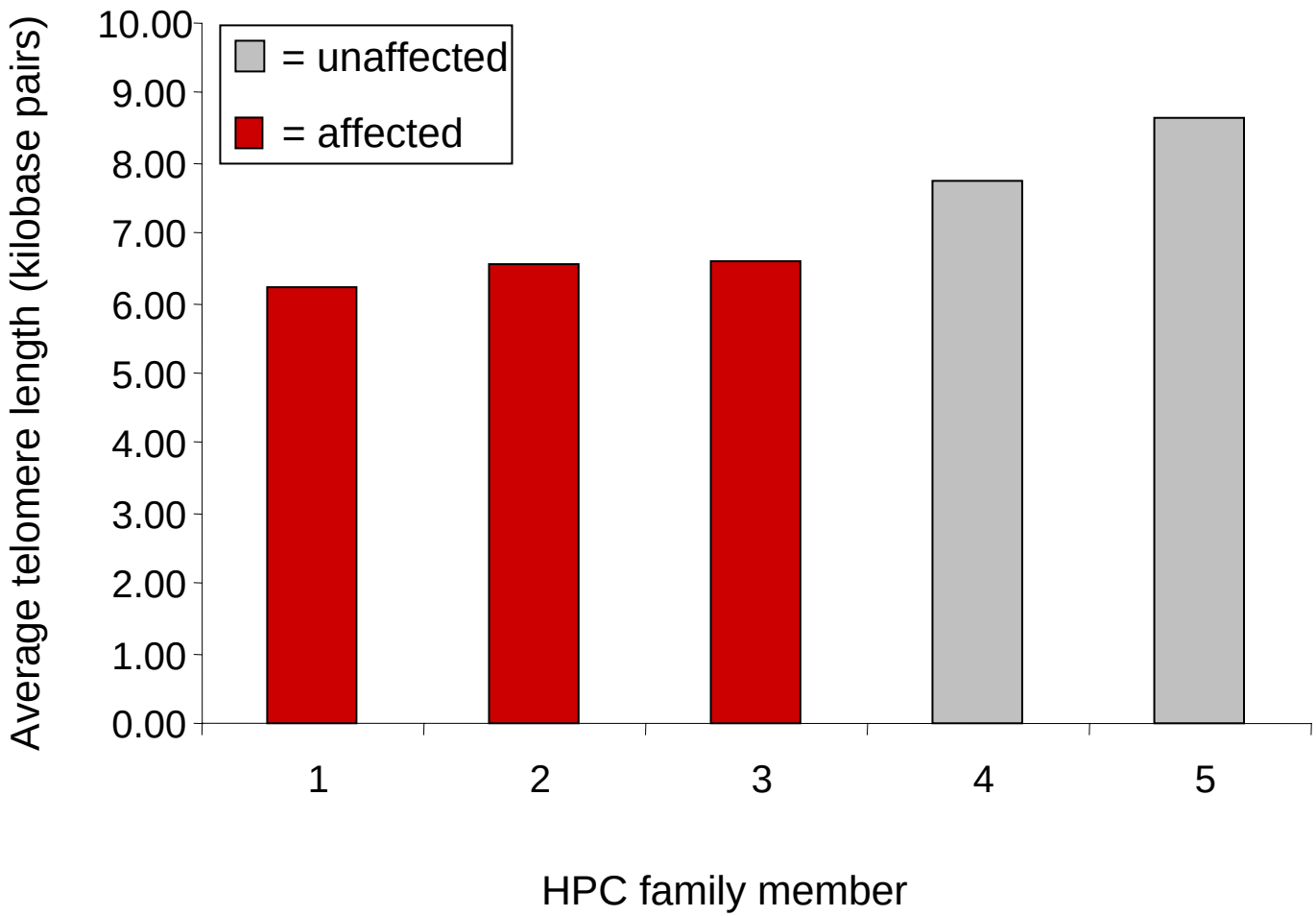
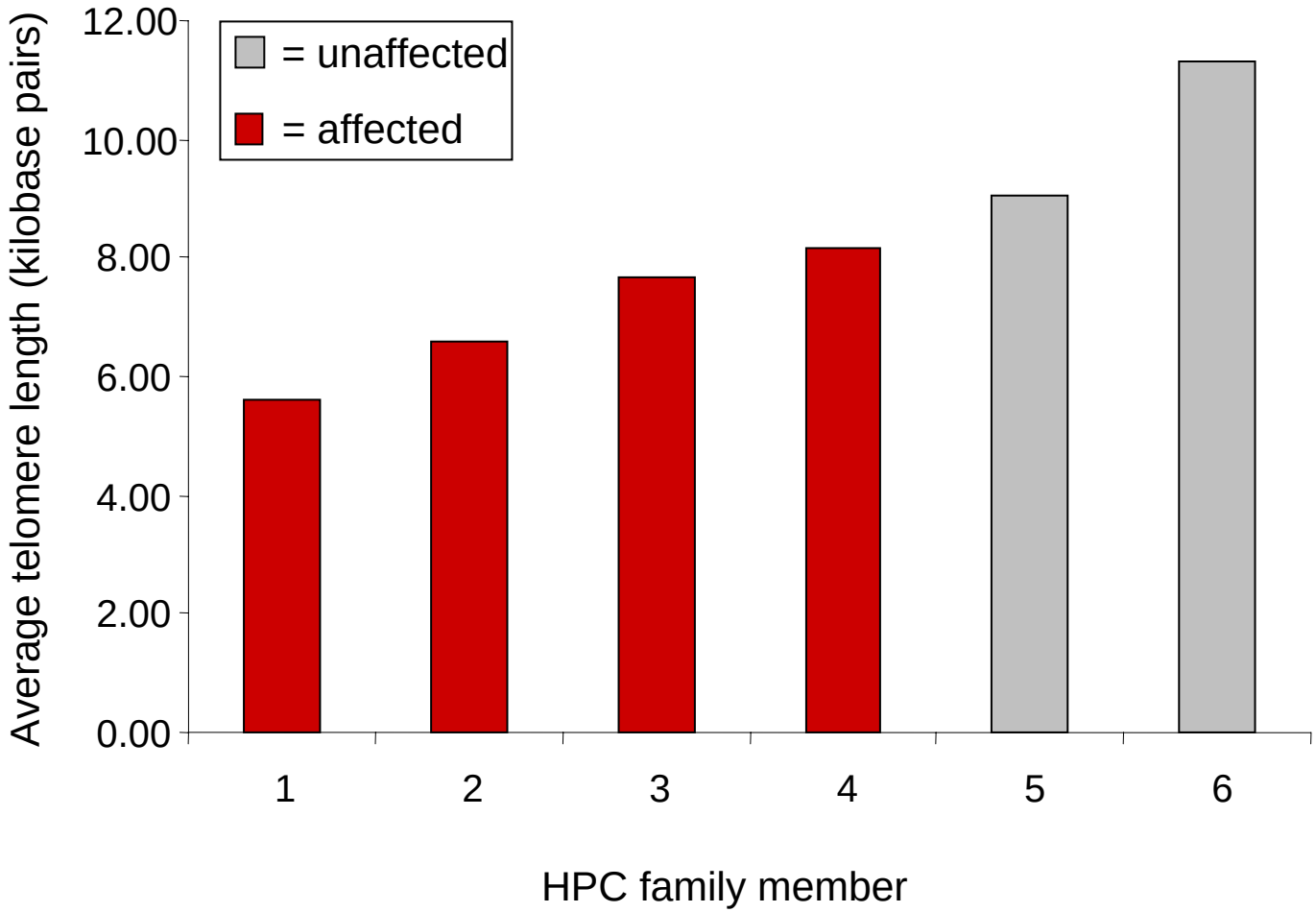
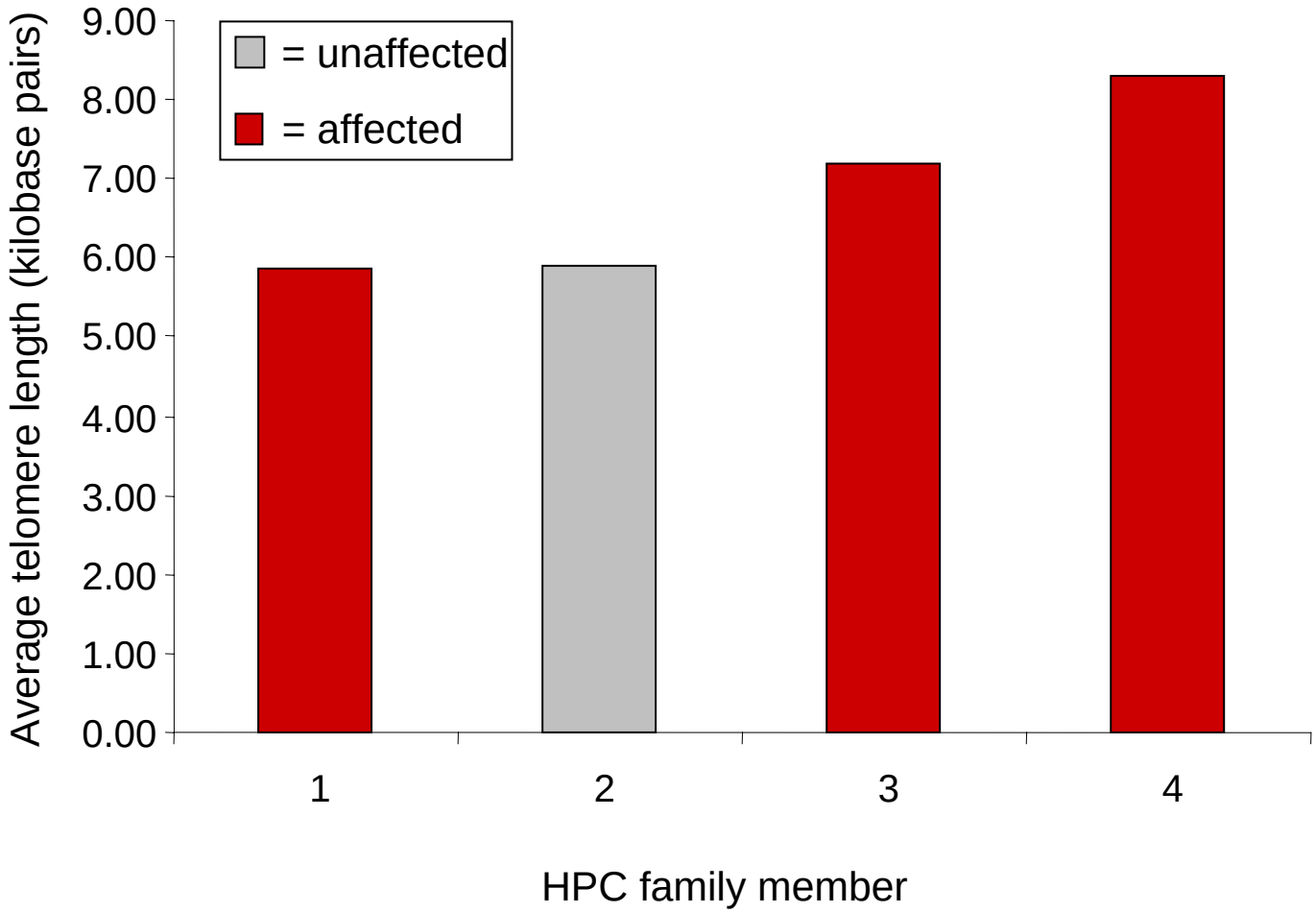


A9: Family #137

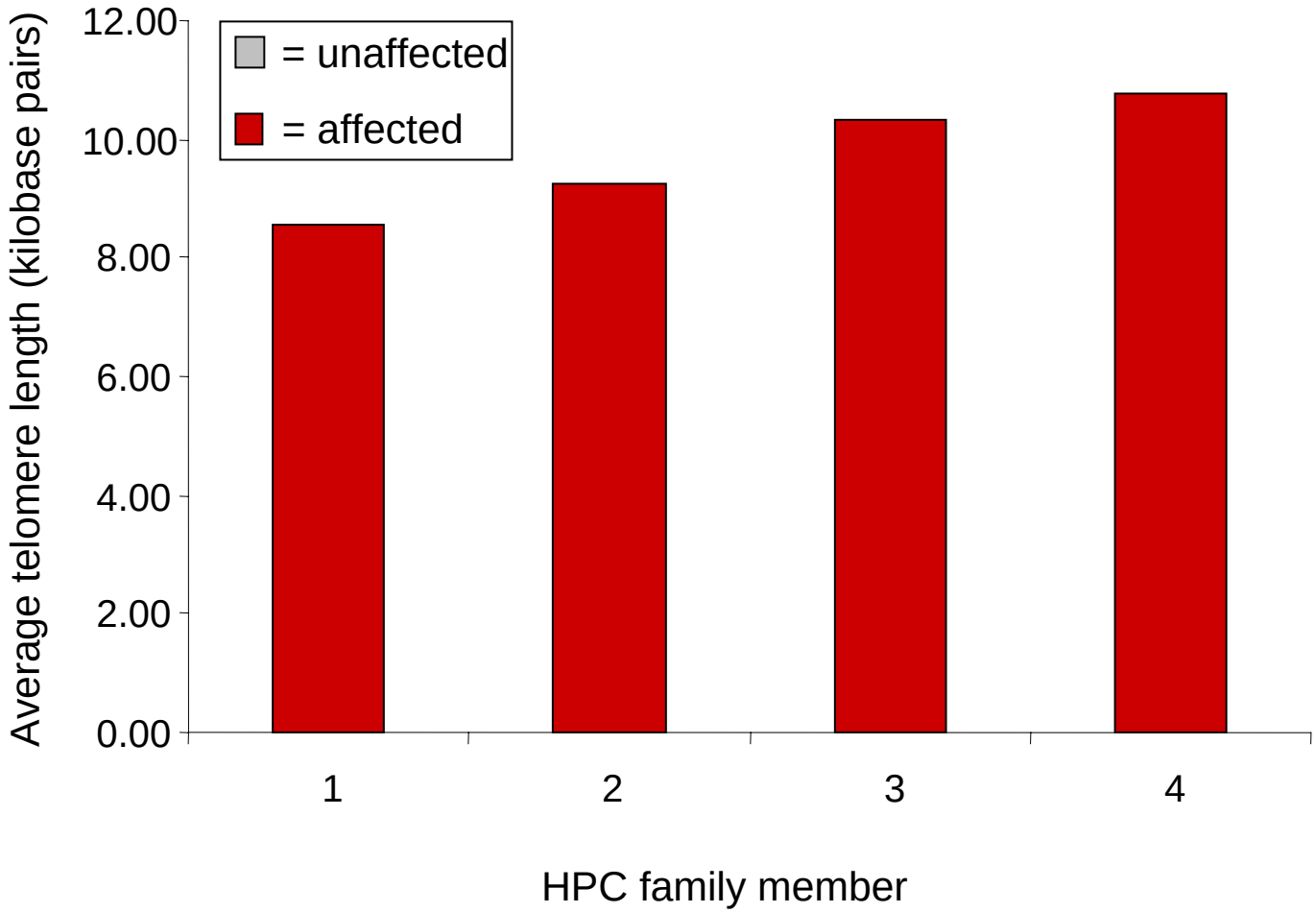


A10: Family #210

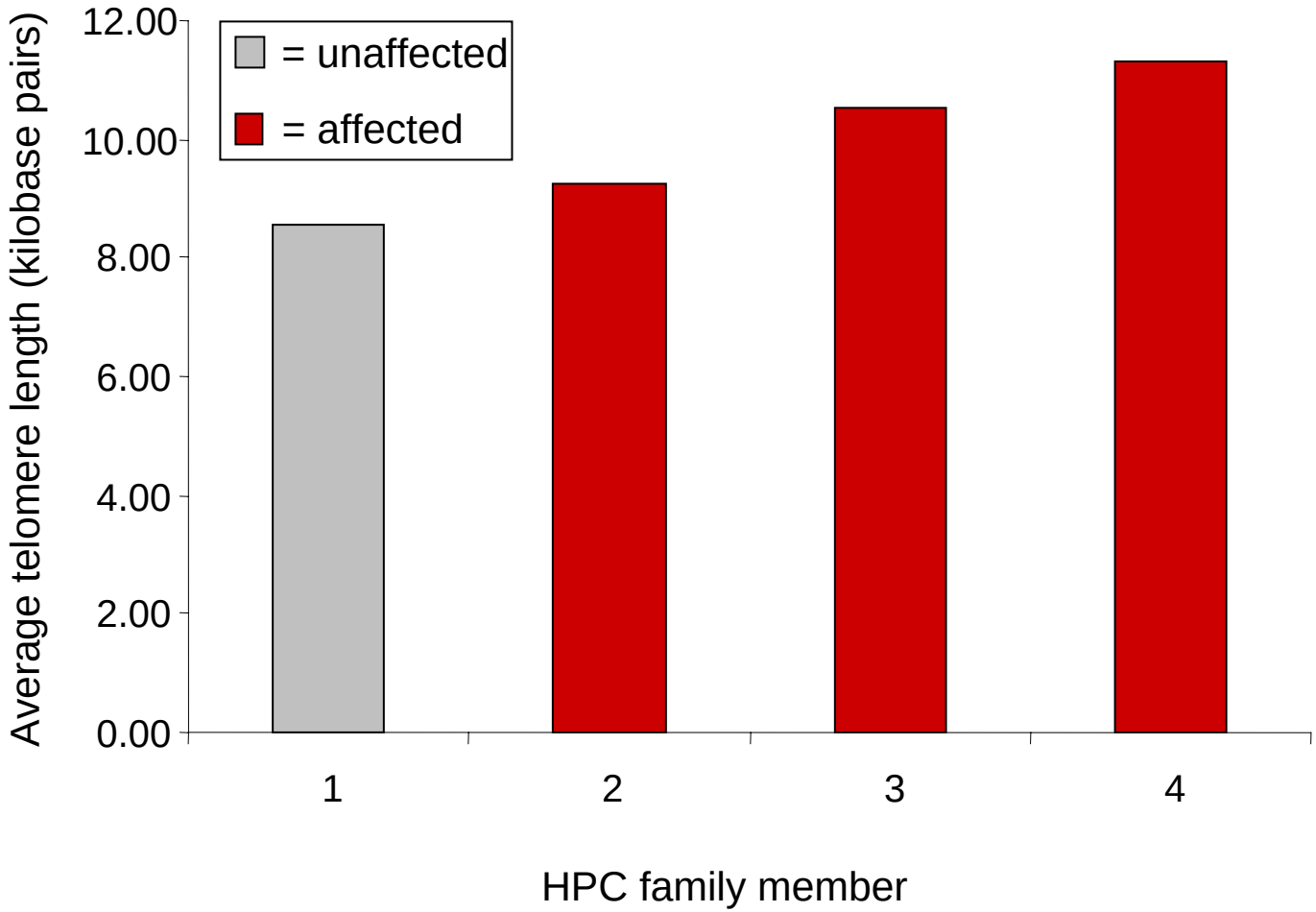
A11: Family #214



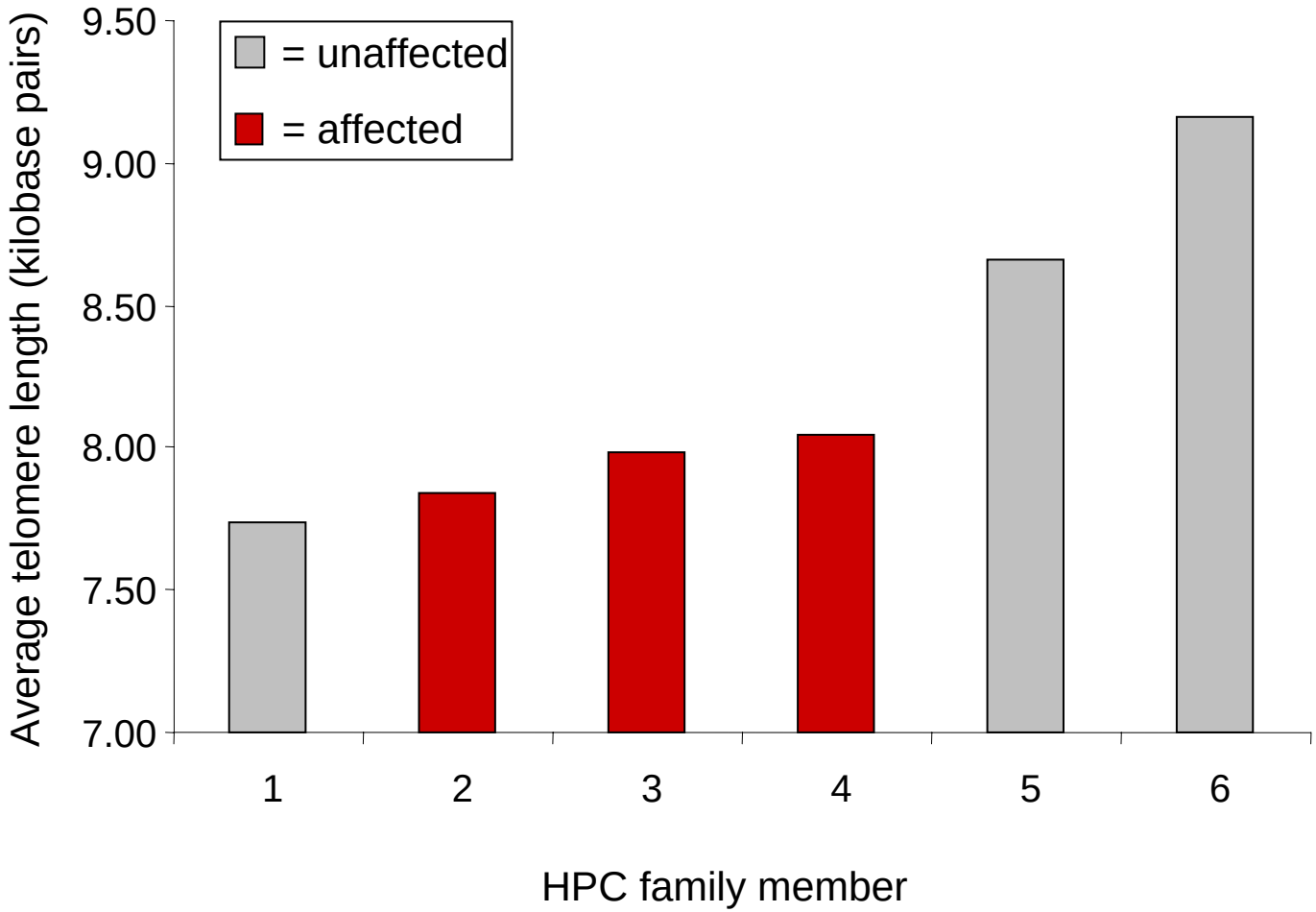
A12: Family #227



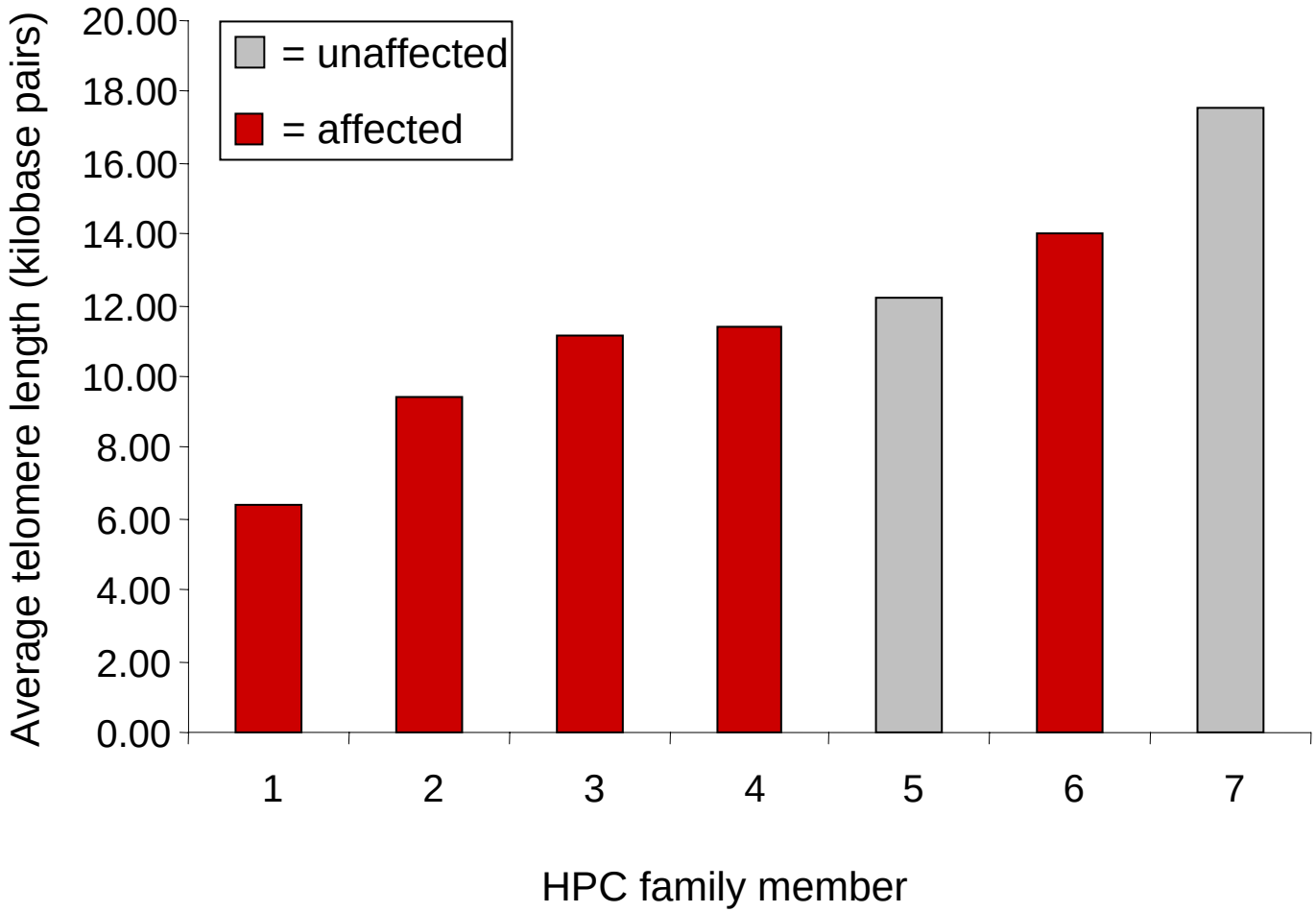
A13: Family #230



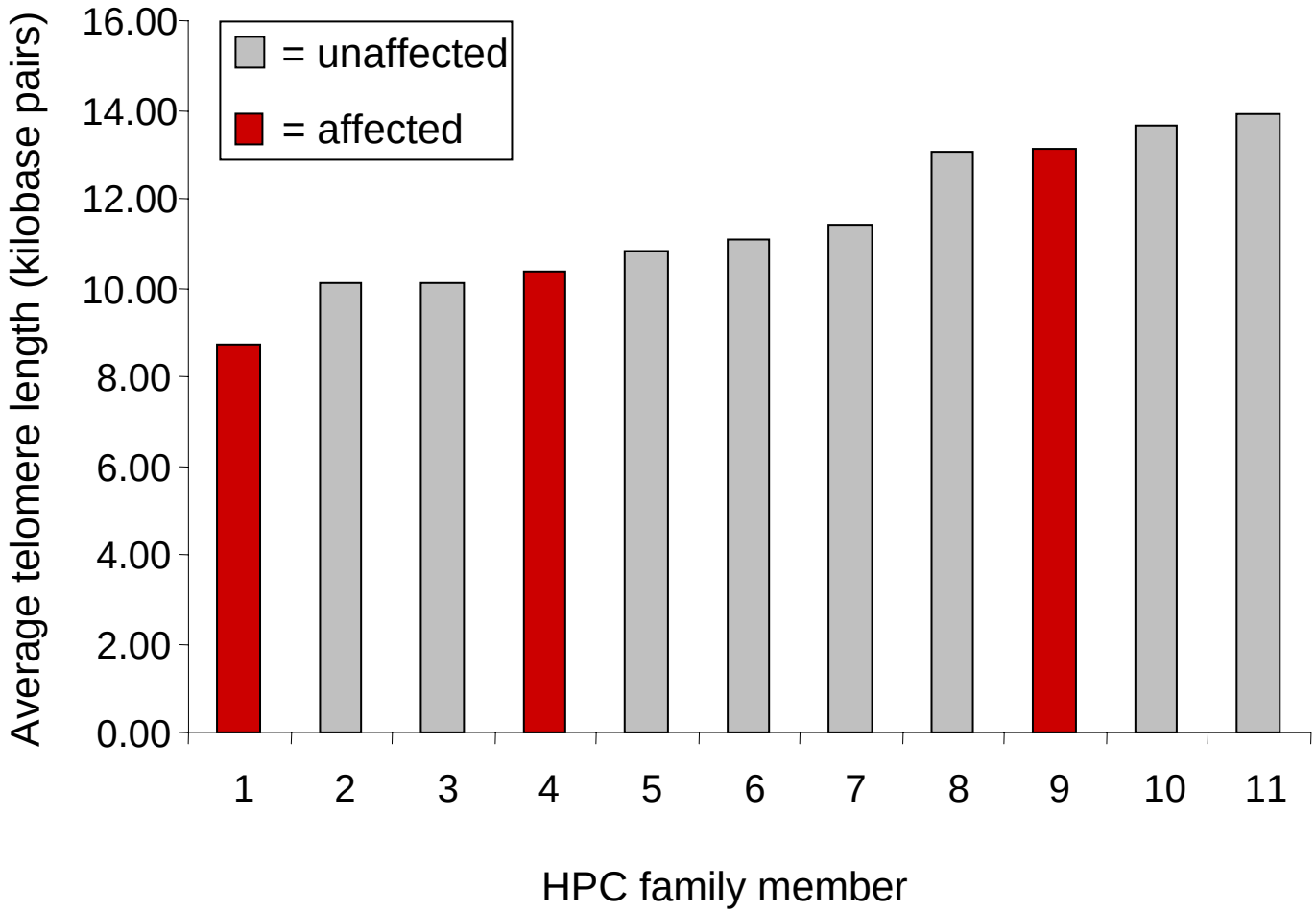
A14: Family #231



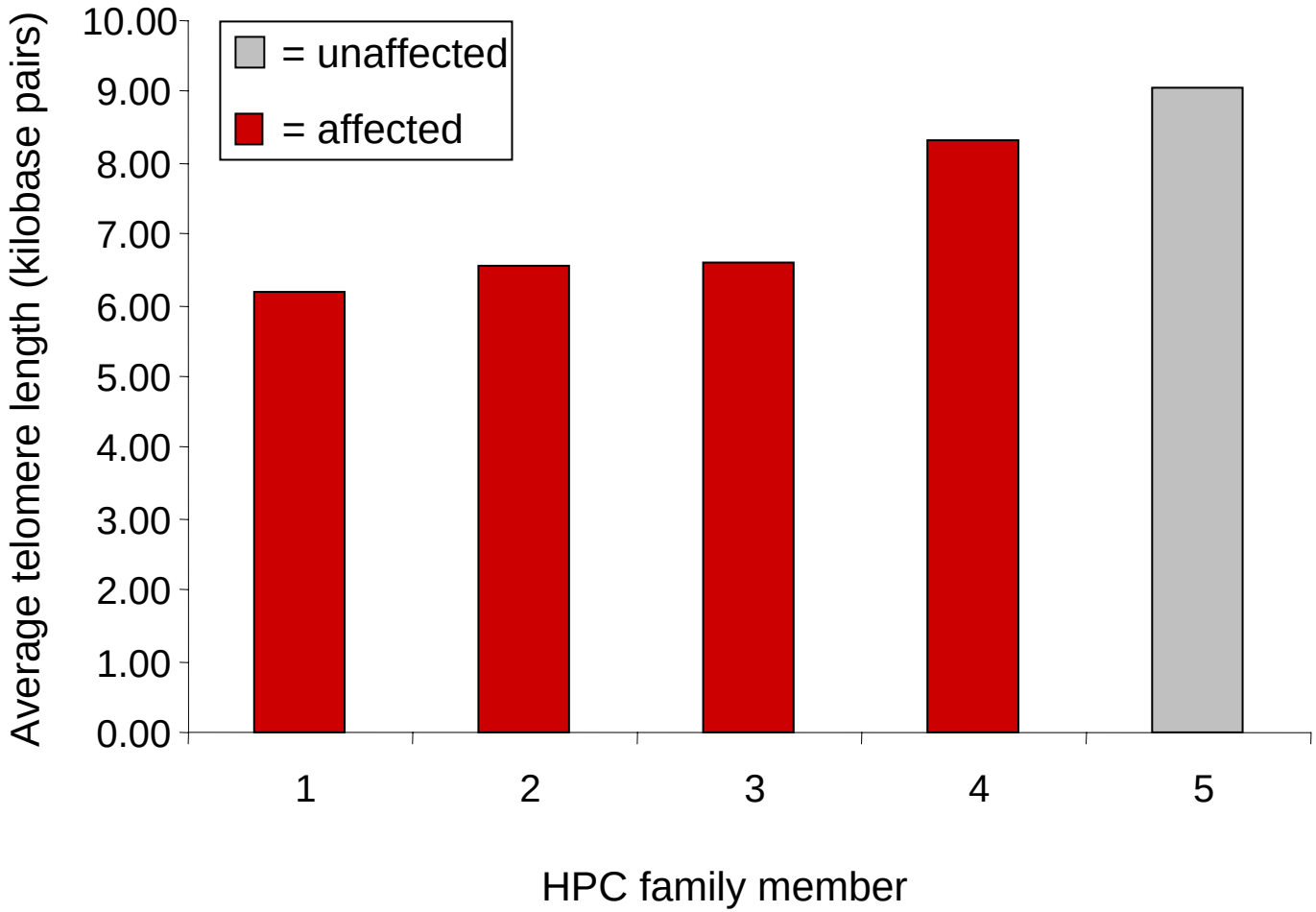
A15: Family #239

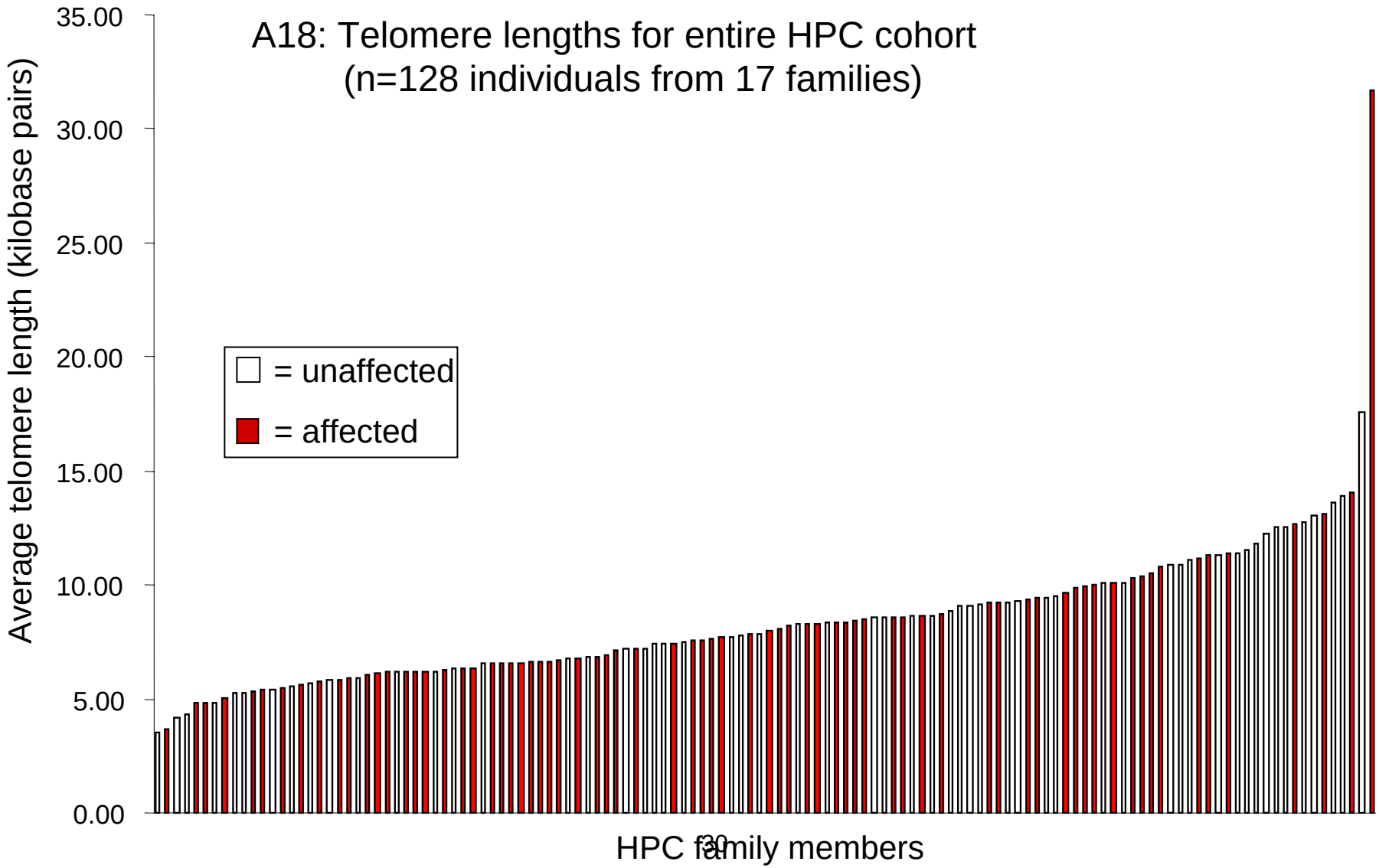


A16: Family #241



A17: Family #244




A18: Telomere lengths for entire HPC cohort
(n=128 individuals from 17 families)
Average telomere length (kilobase pairs)
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
= unaffected
= affected
HPC family members